Kidney International, Vol. 63 (2003), pp. 340–345

Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells

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Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells.

Background. Dialysis patients, often carriers of *Staphylococcus aureus* in their nares, are at high risk of *S. aureus* infections.

Methods. We examined whether RNAIII inhibiting peptide (RIP), which interferes with quorum sensing mechanisms, reduces adherence of *S. aureus* to host cells and to dialysis catheter polymers in vitro. Adherence was tested by spectroscopy using safranin staining, by confocal scanning laser microscopy and by atomic force microscopy.

Results. RIP inhibited bacterial adherence to HaCat and HEp-2 cells and reduced adherence and biofilm formation not only on polystyrene, but also on both polyurethane- and silicone-made dialysis catheters, with a preponderant effect on silicone, to which bacteria were more adherent.

Conclusion. RIP opens a new perspective in anti-*S. aureus* prophylaxis, particularly in dialysis patients.

Staphylococcal infections, particularly by *Staphylococcus aureus*, cause substantial morbidity and mortality in hemo- and peritoneal dialysis patients. *S. aureus* carriage in the anterior nares, a risk factor for infection, is about twofold more prevalent in dialysis patients than in healthy controls [1]. Seventy-five percent of dialysis patients with nasal carriage also carry *S. aureus* on their hands. Molecular typing has revealed that strains from nares, skin and infectious site are identical in 91% of cases [2]. Three-weekly skin punctures of the vascular access site(s) and the use of central venous hemodialysis and peritoneal dialysis polymer-made catheters are other risk factors [3].

Key words: atomic force microscopy, dialysis access, quorum sensing, RIP, infection, hemodialysis, peritoneal dialysis, anterior nares, vascular access, bacteria.

Received for publication April 16, 2002
and in revised form June 25, 2002
Accepted for publication August 28, 2002

A critical pathogenic step is the adherence of *S. aureus* to host cells or polymer surfaces and the subsequent accumulation of toxin-producing bacteria. A novel prophylactic approach is to prevent bacteria from adhering and biofilm from forming, thus lessening the consequent production of disease-causing toxins.

The early stages of biofilm formation to devices such as dialysis catheters involve adherence of bacteria to cellular or matrix surfaces [4]. Further biofilm organization into complex structures is regulated by exchange of chemical signals between bacterial cells in a process known as quorum sensing [5, 6]. The multicellular-like bacterial arrangement is associated with increased resistance to antibiotics [7]. A novel way to prevent colonization and biofilm formation is to interfere with bacterial cell-cell communication that leads to the virulence phenotype [8]. RNAIII inhibiting peptide (RIP) inhibits such cell-cell communication by interfering with quorum sensing mechanisms [6, 8–11]. Prevention of biofilm formation by RIP may therefore not only decrease infection rate, but also preserve the residual bacteria's susceptibility to antibiotics.

To date, two quorum sensing systems have been described in *S. aureus*: one consists of the autoinducer RNAIII-activating protein (RAP) and its target molecule TRAP [6, 8]; the other consists of the peptide pheromone AIP and its receptor AgrC [12, 13]. As the cells multiply and the colony grows, the cells secrete RAP. When RAP reaches a threshold concentration, it induces the histidine phosphorylation of its target molecule TRAP [6]. The phosphorylation of TRAP leads, in a yet unknown mechanism, to the activation of the gene regulatory system *agr*, which encodes for AIP and AgrC [12, 13]. AIP induces the phosphorylation of AgrC, leading to the production of the regulatory RNA molecule RNAIII that induces toxin synthesis [14].

RIP is a seven amino acid-peptide (YSPWTNF), which when synthesized in its amide form, is active at an opti-

mal concentration of 10 μg/10^7 bacteria [10]. Unlike AIPs that require a thiolactone structure for activity [15] and use AgrC as their receptor, RIP is a linear peptide that competes with RAP and inhibits the bacteria from producing toxins [11] by inhibiting TRAP phosphorylation [6, 10]. RIP was already shown to protect animals from keratitis, mastitis, arthritis, sepsis, cellulitis and osteomyelitis, caused by various strains of staphylococci [8, 9]. The present in vitro study examines whether RIP also reduces *S. aureus* adherence both to human cells resembling those of the anterior nares and skin, and to polymers of dialysis catheters.

METHODS

Bacteria

Wild-type *S. aureus* strain RN6390B (ATCC 55620), *S. aureus* RN6911, an *agr*-null strain [14], and nares-isolates of *S. aureus* obtained from 20 patients at the onset of maintenance hemodialysis. The most adherent strain, isolated from patient #7, is termed here as strain 1. The least adherent strain, isolated from patient #20, is termed here as strain 20.

Human cells

HaCat human skin keratinocytes and human epithelial HEp-2 cells were grown at 37°C in a 5% humidified incubator in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS).

Adherence to human cells

S. aureus RN6390B cells were labeled with fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, MO, USA) as described [10]. To test *S. aureus* adherence to human cells, 10^4 HaCat human skin keratinocytes or HEp-2 human epithelial cells were applied to Costar 96-well cell culture polystyrene plates (Corning Inc., Corning, NY, USA) and grown to reach confluency at 37°C in a 5% CO_2 humidified incubator in bicarbonate-buffered DMEM supplemented with 5% FCS. FITC-labeled bacteria [10^6 cells/well in 90 μL phosphate-buffered saline (PBS)] were added to the confluent layer of host cells without serum and with or without 5 μg RIP (synthesized in its amide form by Neosystem, Strasbourg, France). Host and bacterial cells were incubated (30 min, 37°C), washed by PBS and fluorescence determined (485/530 nm) in a Microplate Fluorescence Reader (FL 600; Bio-Tek, Winooski, VT, USA) using KC4 software. For confocal scanning laser microscopy studies, bacterial and human cells were fixed (2.5% formalin), F actin was labeled (Rh-phalloidin) [16] and cells were analyzed (LSM 410; Zeiss, Oberkochen, Germany).

Atomic force microscopy

About 10^{10} cells of early exponential *S. aureus* strain 1 that was isolated from the nares of a dialysis patient (discussed later in this article) were grown with or without RIP (10 μg/10^7 bacteria) at 37°C in culture plates (60 mm polystyrene plates) for three hours. Plates were placed at 4°C overnight. Unbound cells were removed by several washings with PBS, and cells that adhered to the plastic were fixed with 2.5% glutaraldehyde for 15 minutes at room temperature and rinsed with PBS. Bacteria were imaged in PBS using atomic force microscope (BioScope; Digital Instruments, Santa Barbara, CA, USA) in tapping mode. V-shaped silicon nitride cantilevers with a nominal spring constant of 0.01 N/m (Park Scientific Instruments, Sunnyvale, CA, USA) were used for bacterial imaging. Typical resonance frequencies were chosen between 6 and 9 kHz. Images of bacteria were obtained by a serial window narrowing, from the typical 30 μm scale in the first scan to a several hundred nanometers scale in the final scan. Both the initial large scan and following smaller scans were performed in the scan rate range between 0.1 to 1.0 Hz to provide tip velocity of about 2 to 4 μm/s.

Adherence to dialysis catheters

Staphylococcal aureus nasal strains from 20 dialysis patients were grown in broth to early exponential phase. Two hundred microliters of each strain (10^8 cells) were applied in triplicates to 96-well culture polystyrene plates. The strains with highest (strain 1) or lowest (strain 20) adherence to polystyrene were further grown in wells containing a 0.5 cm piece of sterile dialysis catheters made of silicone (chronic silicone catheter; Horizon Medical Products, MA, USA) or polyurethane (single lumen hemodialysis catheter; Gambro, Hechingen, Germany), with or without RIP (100 μg/10^8 bacteria). Cells were grown without shaking (37°C, 3 hours), non-adherent cells removed, adherent cells stained (100 μL 0.1% safranin, 30 seconds), and unbound stain removed. Catheters containing stained bacteria were placed in a new well. Stained cells were dried (30 min, room temperature), 100 μL of 0.1% sodium dodecyl sulfate (SDS) in water was added for 30 minutes, the solution placed in a new well and absorbance read at 490 nm.

Statistical analysis

Statistical analysis was performed using the Student *t* test. Values of $P < 0.05$ were considered significant.

RESULTS

RIP reduces adherence of *S. aureus* to human keratinocytes and epithelial cells

To test whether RIP can prevent *S. aureus* from colonizing host cells, FITC-labeled wild-type *S. aureus* were

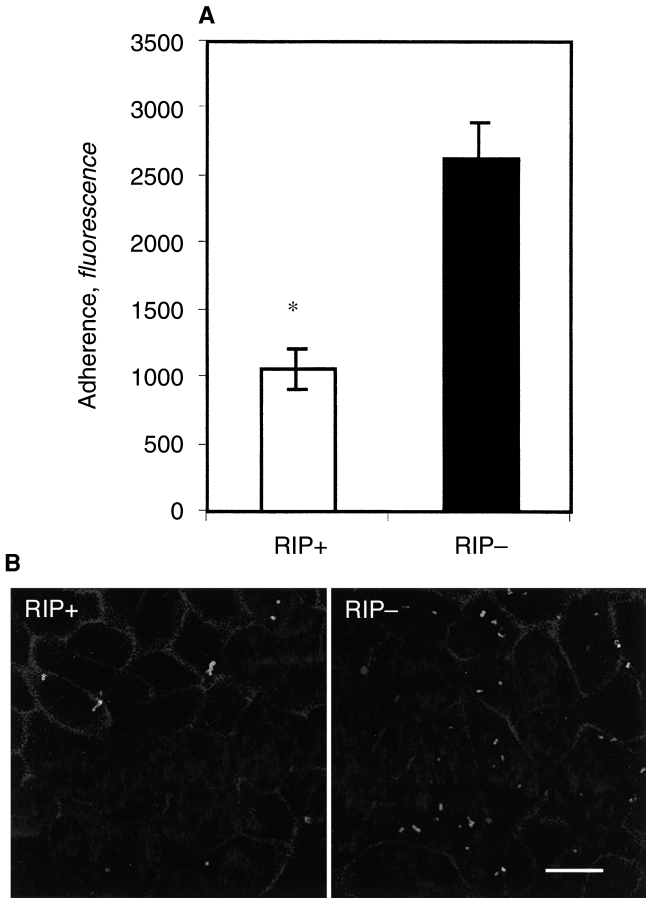

Fig. 1. RNAIII inhibiting peptide (RIP) reduces adherence of *S. aureus* to HaCat cells. (*A*) FITC-labeled bacterial cells were applied to confluent HaCat cells with/without RIP. Adherent fluorescent bacteria were measured at 485/530 nm. $*P < 0.05$. (*B*) F actin was labeled with Rh-phalloidin and cells analyzed by confocal scanning laser microscopy. Bacteria appear as light dots and actin filaments as light lines. Bar = 20 μm.

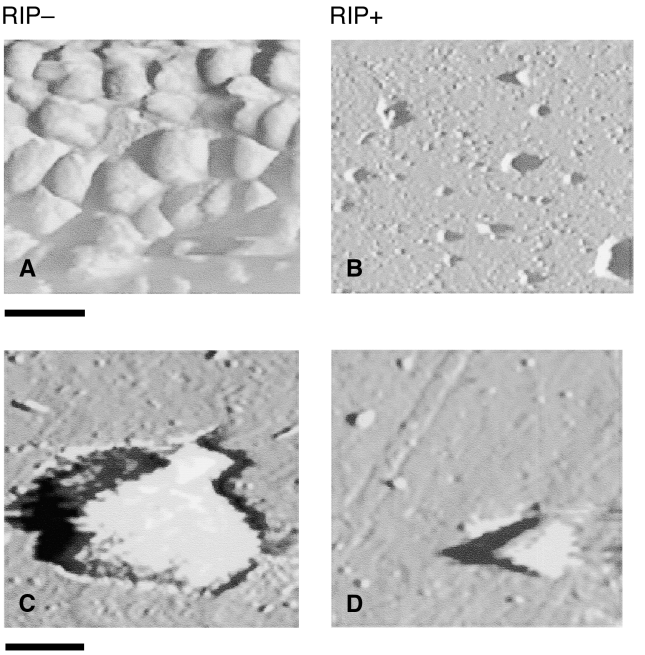

Fig. 2. RIP reduces adherence of *S. aureus* to polystyrene. The atomic force microscopy study used a larger scale to observe number of adherent bacteria in a biofilm (*A*, *B*) and a smaller scale to observe adherence of a single bacterium (*C*, *D*). *S. aureus* clinical strain 1 were applied to polystyrene cell culture plates with RIP (B and D) or without RIP (A and C) and cells observed using atomic force microscopy in tapping mode. Amplitude images were collected and areas of cell attachment were compared. The direction of scan is from left to right. The color code of amplitude images corresponds to the changes in the amplitude of tapping. Top bar (relevant for A and B) corresponds to 2.5 μM. Bottom bar (relevant for C and D) corresponds to 0.5 μM.

incubated with/without RIP and with a confluent layer of keratinocytes (HaCat) or epithelial cells (HEp-2). Figure 1 shows the adherence of bacteria as determined by measuring fluorescence level (Fig. 1A) and as observed by confocal scanning laser microscopy (Fig. 1B). RIP significantly ($P < 0.05$) reduced the number of *S. aureus* cells adhering to HaCat cells. Similar results were obtained for HEp-2 cells (not shown).

RIP reduces catheter adherence and biofilm formation of a strongly adherent *S. aureus* strain isolated from the nares of a dialysis patient

To test whether RIP can reduce biofilm formation of *S. aureus* on catheter polymers, strains isolated from the nares of 20 hemodialysis patients were used and their adherence was first studied to polystyrene by growing bacteria for three hours in polystyrene wells and determining the number of adherent bacteria by safranin staining. These experimental conditions allow the bacteria to adhere to the plastic surface and then form a biofilm. The 20 *S. aureus* nares strains presented variable degrees of adherence to polystyrene, with the most adherent strain (strain 1) adhering fivefold more than least adherence strain (strain 20). Therefore, strain 1 was used to study adherence to plastic polymers in more detail by atomic force microscopy (AFM), which allows the examination of local adherence properties of a single bacterium [10]. Figure 2 A and B show a general view of the biofilm formed on polystyrene by strain 1 with or without RIP, as studied by AFM. An almost confluent bacterial layer was seen in the absence of RIP (Fig. 2A), contrasting with only very few remaining adherent cells in the presence of RIP (Fig. 2B). In the presence of RIP, these few bacteria also attached less strongly, as observed by the amplitude images of a larger scale (Fig. 2 C, D). Amplitude mode, which allows the steep parts of an object and particularly object borders to be emphasized, is illustrated in Figure 2C, a bacterial cell in the absence of RIP exhibiting a flat upper part (amplitude of tapping is almost unperturbed) that gradually drops to the surface. The total contact area between bacterium and surface is much larger than the area of the upper flat part. In

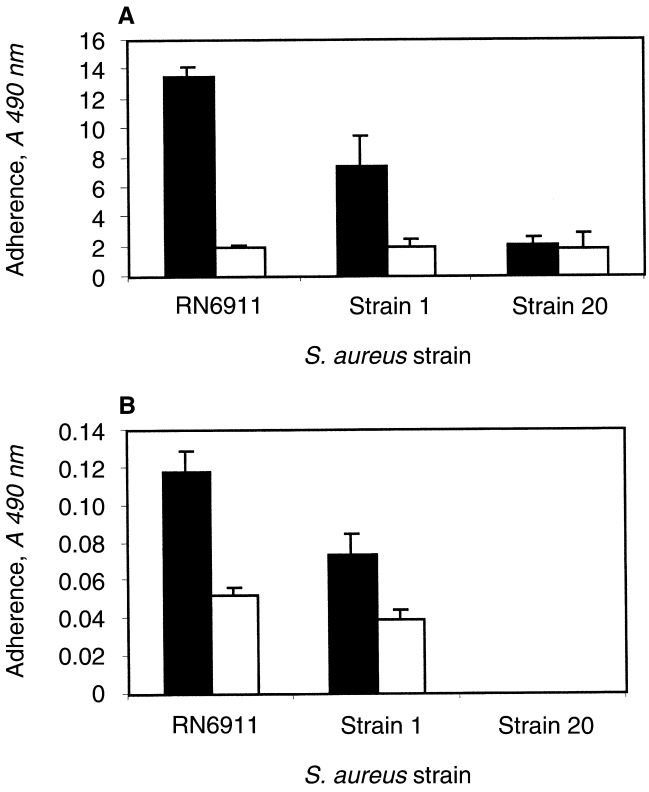

Fig. 3. RIP reduces adherence of *S. aureus* to silicone (*A*) and to polyurethane (*B*). Cells (*S. aureus* RN6911, strain 1 and strain 20) were incubated with silicone- or polyurethane-made dialysis catheter fragments in the presence (□) or absence (■) of RIP. Adherent bacteria were stained with safranin and absorbance was determined at 490 nm.

addition, the bacterial cell has some protrusions scattered over a large surface. In contrast, in the presence of RIP (Fig. 2D), the bacterium has no visible protrusions and transition from the upper part to the attached one is very steep, making the cell appear much smaller. Moreover, the total area of bacterial adherence without RIP (Fig. 2C) is at least four times larger than that of the cell treated by RIP (Fig. 2D). These results indicate that RIP decreases not only the number of attached bacteria, but also the contact area between the single bacterium and the polystyrene surface, suggesting reduced adhesion forces in the presence of RIP.

Further studies were done on the adherence of strain 1 and strain 20 to more clinically relevant polymers, that is, silicone and polyurethane dialysis catheters. Bacteria in the early exponential growth phase were added to pieces of these polymers, adherent bacteria were stained with safranin and absorbance was determined. As shown in Figure 3, adherence of strain 1 was approximately tenfold greater to silicone than to polyurethane ($P < 0.05$). Importantly, adherence of strain 1 both to silicone and to polyurethane was significantly reduced in the presence of RIP ($P < 0.05$). On the other hand, adherence of strain 20 to silicone was relatively low to begin with, and RIP did not further reduce its adherence in the concentration range tested. Adherence of strain 20 to polyurethane was below detection level. These results suggest that the effect of RIP is especially pronounced in highly adherent strains.

To test if RIP affects *S. aureus* adhesion via *agr*, adherence assays were carried out on *S. aureus* RN6911. This strain is an *agr*-null strain and thus would be expected to be highly adherent [14, 15]. As shown in Figure 3, RIP significantly reduced adherence of *S. aureus* RN6911, suggesting that RIP affects adherence by regulating gene loci that are independent of *agr*.

DISCUSSION

Current strategies to prevent *S. aureus* infections in dialysis patients consist of improved hygiene, the use of anti-staphylococcal compounds either systemically (such as rifampin) or topically (for example, mupirocin application to the nares [17] or the catheter exit site), impregnation of catheters with antimicrobial compounds, use of an "antibiotic lock" to fill the catheter lumen during the interdialytic interval [3] and, more recently, vaccination [18]. The present in vitro study examines a new strategy: to impair adherence of *S. aureus* to critical host cells and to dialysis catheters by using RIP.

We found that RIP decreases bacterial adherence to human keratinocyte line HaCat. As anterior nares are lined by keratinocyte-like cells [1], our results suggest that RIP may be used for reducing or eradicating nasal carriage. Because the vast majority of *S. aureus* strains isolated from infected sites are identical to those isolated in the anterior nares and on the skin [2], impairing bacterial adherence to anterior nares or skin cells by RIP may be of critical importance to prevent the infectious cascade. Studies are needed to compare the efficacy of the nasal application of either RIP or mupirocin, the latter being currently used for long-term eradication of nasal carriage of *S. aureus* in hemo- and peritoneal dialysis patients [17].

RNAIII inhibiting peptide also decreases adherence and biofilm formation of *S. aureus* strains isolated from the nares of hemodialysis patients to several plastic materials and in particular to dialysis catheters made of silicone or polyurethane. The high degree of inhibition of bacterial adherence to silicone by RIP is of interest, as long-term catheters for hemo- and peritoneal dialysis are usually made of silicone. One potential application is to coat dialysis catheters with RIP. Coating both the external and the luminal surface of catheters may be more effective than coating only the luminal surface, as is the case with the "antibiotic lock" technique, where a solution containing either heparin or citrate and an antimicrobial compound is installed in the lumen of the hemodialysis catheter at the end of dialysis [3]. The latter

technique may be useful but is not designed to prevent *S. aureus* infections initiating at the catheter exit site.

Different technical approaches were used in this study to assess adherence properties of *S. aureus.* Fluorescence measurement and confocal scanning laser microscopy both indicated that significantly less bacteria adhered to host cells in the presence of RIP, spectroscopy with safranin staining showed that significantly less bacteria adhered to clinically relevant catheter polymers when RIP was present, and finally the more sophisticated atomic force microscopy was used, showing at the single bacterium level that RIP decreases the contact surface and therefore adhesion forces between *S. aureus* and polystyrene polymers. The fact that the results reached by these different technical approaches converge strengthens our conclusion on the inhibitory effect of RIP on adherence properties of *S. aureus.*

Adherence experiments were done also in the presence of an inactive form of RIP (YSPWTNF which was synthesized without an amide [10]). Inactive RIP did not reduce bacterial adhesion, suggesting that RIP (produced in the amide form) acted specifically (discussed later in this section) to reduce adhesion and not through fortuitous charge interactions.

RIP competes with RAP on the phosphorylation of TRAP, leading to inhibition of TRAP phosphorylation and *agr* expression [6], resulting in the suppression of RNAIII-regulated toxin synthesis [11]. Down-regulation of *agr* is known to lead to up-regulation of bacterial adhesion proteins [14, 15, 19]. However, as shown here and in Gov et al [10], RIP decreases bacterial adhesion. This suggests that RIP affects other factors in addition to *agr*, resulting both in decreased toxin production and decreased adhesion properties. To test for this hypothesis, we tested the effect of RIP on adherence of *S. aureus* RN6911, which is an *agr* null strain. As shown here, RIP reduces bacteria adherence also in the absence of *agr*, suggesting that RIP effects bacterial adhesion through gene loci that are independent of *agr*. This phenomenon is not surprising in view of the fact that TRAP, which is a target molecule of RIP, was first described as a quorum sensing transducer of *S. aureus* [6], but was then suggested to be a more global responder to stress [20], suggesting that it too acts on other systems in addition to *agr*.

RIP has been shown to inhibit pathogenesis of every strain tested thus far [9], as opposed to AIPs that are strain-specific due to *agr* variability [13, 21]. This can be explained by the fact that unlike AIPs and AgrC, TRAP is a highly conserved protein in staphylococci. Indeed, RIP inhibits TRAP phosphorylation, adherence, biofilm formation and pathogenesis also in *S. epidermidis*, where TRAP is highly homologous to that of *S. aureus* [22]. Therefore, RIP can be used as a global suppressor of staphylococci, and not be strain-specific as the AIPs.

The present results from an in vitro study obviously require confirmation in vivo. Using a rat model with subcutaneous implantation of drug sensitive (MSSA) or drug resistant (MRSA) *S. aureus*-infected Dacron grafts, our data indicate that RIP significantly reduces bacterial load on the graft (Balaban et al, manuscript in preparation).

All together, our studies on adherence of *S. aureus* to host cells and clinically relevant polymer materials clearly demonstrate that RIP reduces not only the number of adherent bacteria, but also bacterial adhesion properties. These results support our hypothesis that RIP may be used to coat patient anterior nares and dialysis catheters to prevent staphylococcal colonization and consequent infections.

ACKNOWLEDGMENTS

We thank Dr. H.W. Van Landuyt and Dr. B.Z. Gordts for their help in microbial identification and storage, and Dr. A. De Vriese for manuscript review.

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